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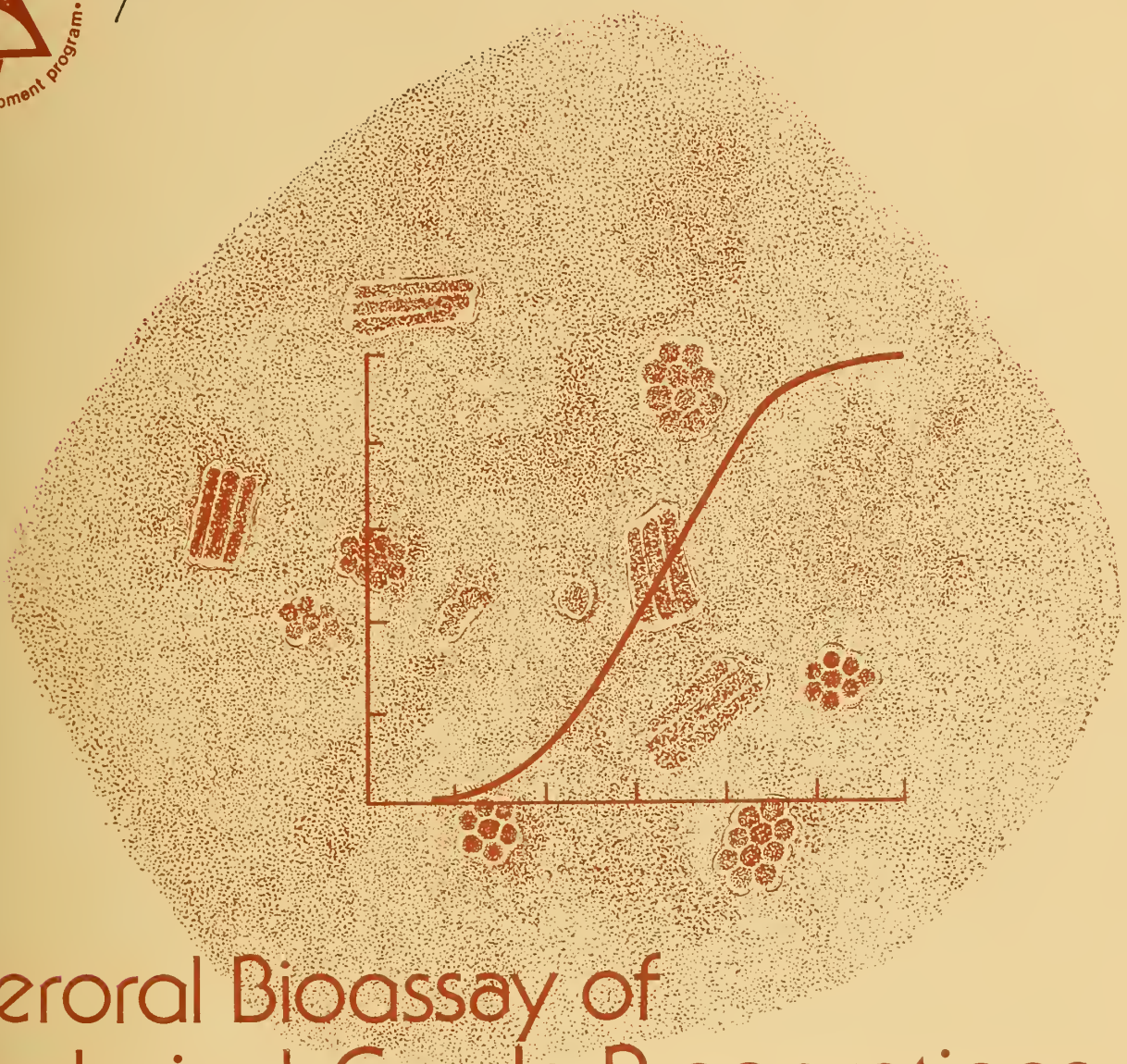

Peroral Bioassay of Technical-Grade Preparations of the Douglas-Fir Tussock Moth Nucleopolyhedrosis Virus (*Baculovirus*)

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Contents

PERORAL BIOASSAY OF TECHNICAL-GRADE PREPARATIONS OF THE DOUGLAS-FIR TUSSOCK MOTH NUCLEOPOLYHEDROSIS VIRUS (BACULOVIRUS)

Reference Abstract

Martignoni, Mauro E., and Paul J. Iwai.
1977. Peroral bioassay of technical-grade preparations of the Douglas-fir tussock moth nucleopolyhedrosis virus (*Baculovirus*). USDA For. Serv. Res. Pap. PNW-222, 12 p., illus. Pacific Northwest Forest and Range Experiment Station, Portland, Oregon.

The potency of industrial preparations of viruses for control of injurious insects can be estimated reliably only by means of biological assay. We describe a simple, yet sensitive peroral bioassay procedure for technical-grade preparations of nucleopolyhedrosis virus. This procedure has been accepted by the Forest Service and the U.S. Environmental Protection Agency as the standard method for assaying industrial virus preparations for control of the Douglas-fir tussock moth.

KEYWORDS: Bioassay, nucleopolyhedrosis, virus (insect), virus (-insecta, virus (-forest pest control, insect diseases, diseases (insect), insect damage control (forest), biological control (-forest pests, Douglas-fir tussock moth, *Orgyia pseudotsugata*.

RESEARCH SUMMARY

Research Paper PNW-222

1977

Gravimetric, chemical, and particle-counting procedures are available for estimating the approximate activity of preparations of insect viruses. Only by biological assay, however, can the actual potency (activity or infectivity titer) of such preparations be determined. To minimize variation and to increase the reproducibility of potency assessments, we describe a procedure designed to cope specifically with the usual complexity of measuring a biological stimulus (nucleopolyhedrosis virus) in a living host (Douglas-fir tussock moth). This bioassay, several years in development, has been tested repeatedly in its present format. It has a high degree of reproducibility. The final protocol, as presented here, has been accepted by the U. S. Environmental Protection Agency and by the Forest Service as part of the production control procedure for industrial preparations of nucleopolyhedrosis virus of the Douglas-fir tussock moth.

Introduction

Finney (1971) defines bioassay as follows: "In its widest sense, the term means the measurement of the potency of any stimulus, physical, chemical or biological, physiological or psychological, by means of the reactions that it produces in living matter." Although the biological method of measuring stimuli is usually cumbersome and time-consuming, it is adopted as a rule when a physical or chemical measure of the stimulus does not provide reliable information on its biological activity. This is especially true for technical-grade preparations, when a physical or chemical measure of the active ingredient can be difficult, if not impossible, to obtain.

Biological assay, because of its exacting requirements and technical complexity, appears to have been shunned by most workers as a method for quantifying technical nucleopolyhedrosis virus preparations for field use. A study of the literature shows that the number of polyhedral inclusion bodies (PIB) per unit weight or volume of nucleopolyhedrosis virus preparations, or per unit treated area, is the most commonly used "measure" of the potency of such preparations. In the United States, for instance, nucleopolyhedrosis virus preparations of *Heliothis* sp. on cotton are measured in *viral units* (1 VU = 10^9 PIB) and in *larval equivalents* (1 LE = 6 x 10^9 PIB = 6 VU) (Allen 1967; Woodall and Ditman 1967; Pinnock 1975). PIB counts of technical preparations, by microscopic as well as electronic procedures, can be quite inaccurate, however. Variations in counts among operators and among laboratories are large (Martignoni and Iwai 1968; C. M. Ignoffo, *quoted in* Burges and Thomson 1971). Furthermore, because of the considerable variation in methods of storage of technical nucleopolyhedrosis virus preparations (packaging, moisture, temperature, age), PIB counts cannot be translated reproducibly into units of activity of these preparations. Not surprisingly, dose computations based on PIB counts have been eyed with suspicion for some time. Pinnock (1975) states: "Probably the greatest imprecision in the reporting of virus trials for crop protection occurs in the estimation of the dosage of virus applied. Larval equivalents (LE) are the most frequently used measure of dose, yet the measure is highly variable,. . . ." After a statistical study of PIB counts obtained by electronic transducer and through conventional microscope counting procedures, we concluded ". . . that the polyhedron counts of suspensions prepared with raw tissue homogenates are unreliable, and that the biological assay of such suspensions provides a more consistent estimate of the potency of such preparations (e.g., LD_{50} in nanograms of preparation per gram of larval weight)" (Martignoni and Iwai 1968). Yearian (1975) pleads for standardization of the activity of *Baculovirus* preparations used in the field. Finally, and unequivocally, the U. S. Environmental Protection Agency (1975) requires that quantitation of nucleopolyhedrosis and granulosis virus preparations be based on bioassays rather than counts of inclusion bodies.

History

During the late sixties and the seventies, the U. S. Forest Service and private industry developed a strong interest in nucleopolyhedrosis virus preparations for the control of the Douglas-fir tussock moth, *Orygia pseudotsugata*. Pilot-plant production methods, laboratory testing procedures, and field application techniques were developed and tried. The development and testing of biological

assay procedures was part of this broad effort to manage an economically important insect pest by means of a viral product.

In 1966, the first pilot-plant scale batches of a technical nucleopolyhedrosis virus preparation were produced for the Forest Service by industry. Samples of these batches were assayed in our laboratory by a microfeeding technique, which permits individual inoculation of larvae with glass microcatheters. We used 75±5-mg larvae. The rearing temperature was $30^{\circ}\pm0.5^{\circ}C$, and the holding period was 12 days. The dose unit was nanograms (dry weight) of sample; the LD_{50} was expressed in nanograms per larva. The details of the procedure were described for the first time in 1967, and they remained essentially unchanged through several revised editions of the pamphlet (Martignoni 1975). The potency of each batch of technical nucleopolyhedrosis virus preparation produced since 1966 was measured in our laboratory by the microfeeding bioassay technique. In 1974, we submitted this procedure to the Registration Division of the U.S. Environmental Protection Agency, as part of the U.S. Forest Service application for a temporary permit for experimental use of the nucleopolyhedrosis virus preparation in Pacific Northwest forests (EPA Temporary Permit No. 27586-EXP-2G, June 4, 1974).

The intense interest of the Forest Service and of private companies in viral preparations for the control of Douglas-fir tussock moth outbreaks will lead, eventually, to industrial-scale production of virus and to routine bioassay by the industry and its customers. Because the microfeeding technique requires certain instruments and technical skills available only in few research laboratories, we decided to develop an alternate bioassay procedure. This new procedure should be simple, yet sensitive; it should not require specialized instrumentation other than that available in most biological laboratories.

During 1974 and 1975, we developed and tested a "diet-surface treatment" (DST) bioassay procedure similar in principle to that used by Ignoffo (1966) for the bioassay of the nucleopolyhedrosis virus of *Heliothis zea*. In a DST procedure, the viral preparation is placed directly on the surface of the diet, in an appropriate rearing container, rather than being blended with the diet. Procedures in which the active agent is blended with the diet (e.g., the bioassay of *Bacillus thuringiensis* δ-endotoxin; Dulmage et al. 1976) are known as "diet-incorporation" techniques. The major difference between the *Heliothis* DST procedure and ours is the age of the test larvae. We use recently molted, second-instar *O. pseudotsugata* larvae. Ignoffo's procedure prescribes neonate *H. zea* larvae. We use second-instar larvae, because neonate larvae of *O. pseudotsugata* are too delicate. Their survival rate to second instar, on an artificial diet, is usually less than 80 percent, and it can be as low as 60 percent. The causes of this high mortality rate are unknown. No cases of nucleopolyhedrosis have been observed in our bioassay strain. In contrast, the survival rate of second-instar larvae to the third instar, on artificial diet, is usually 100 percent and rarely lower than 95 percent.

The DST bioassay procedure described here was submitted to the Registration Division of the U.S. Environmental Protection Agency, as part of the General Product Chemistry section of the Forest Service application for registration of "TM BioControl-1," the nucleopolyhedrosis virus of the Douglas-fir tussock moth (EPA Registration No. 27586-1, August 11, 1976). Currently,

this bioassay procedure is the official method for testing the activity of nucleopolyhedrosis virus preparations for the control of the Douglas-fir tussock moth. Because of the assay's sensitivity, it is also suitable for environmental monitoring programs and for susceptibility studies in populations of *O. pseudotsugata*.

A method for the standardization of the potency of technical-grade nucleopolyhedrosis virus preparations has been submitted to the U.S. Environmental Protection Agency. We will present the details of the method, its reproducibility, and its sensitivity in a future publication.

Standardized Bioassay

I. PREPARATION OF THE TEST CUPS

A. Materials and equipment
   1. A supply of mixed, warm liquid diet 65-W-PEN (Thompson and Peterson[1/]); minimum amount: 300 ml (sufficient for 240 cups).
   2. 10-ml wide-tip sterile disposable pipets
   3. Disposable analyzer cups (2-ml) with write-on wings, as many as required (one per larva) (Scientific Products diSPo® automatic analyzer beakers, B2715).[2/] The cups are exposed for 30 minutes in a UV sterilizer before being used.
   4. Sterile paper tissues
   5. Aluminum foil
   6. Adhesive labels
   7. Rubber aspirator, for pipets
   8. Thermometer (for 60°C water bath)
   9. Hot plate, with water bath (60°C)
   10. Sterile hood
   11. Ultraviolet sterilizer

   Note: The diameter of the analyzer cup at the level of the 1-ml mark is 11.8 mm and the surface area 109.4 mm^2.

B. Procedure
   1. Place the container with the mixed warm diet in the water bath, at 60°C. Fill one 10-ml pipet with diet. With a sterile paper tissue, wipe the excess diet off the exterior of the pipet tip.
   2. Place the tip of the pipet at the level of the 1-ml mark of the analyzer cup, without touching the wall of the cup. Dispense the diet to that mark. Take your time and be precise.
   3. Discard all analyzer cups with air bubbles on the surface of the diet or with streaks of diet on the wall.
   4. Let the cups air dry under a sterile hood for a minimum of 1 hour. Wrap batches of 20 analyzer cups with aluminum foil, label, and date. Refrigerate at 4°C. Preferably, these cups should be used within 1 week, but they can be stored and used for up to 2 weeks.

   Note: For filling larger batches of test cups (600 or more), we recommend an automatic dispensing device.

[1/] Thompson, Clarence G., and Linda J. Peterson. How to rear the Douglas-fir tussock moth. Pacific Northwest Forest and Range Experiment Station, Corvallis, Oregon 97331. [In press].

[2/] The use of trade, firm, or corporation names in this report is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

II. SELECTION OF SECOND-INSTAR LARVAE

A. Materials

1. Sterile toothpicks
2. Disposable petri dishes, 100- x 15-mm
3. An adequate supply of late first-instar larvae of *O. pseudotsugata*, usually twice the number of larvae needed for the bioassay; *O. pseudotsugata* larvae reach the second instar about 7 days after hatching, at $21^{\circ} \pm 2^{\circ}$C, on diet 65-W-PEN, in plastic petri dishes.

B. Procedure

1. The day before the test: at 4:00 p.m., select and discard all second-instar larvae present in an appropriate batch of stock larvae (6-7 days after hatching). Only first-instar larvae should remain in the rearing containers.
2. The day of the test: at 9:00 a.m., select the required number of second-instar larvae from the batch screened the previous day. The second-instar age of these larvae is 1-17 hours. Place 20 second-instar larvae to a petri dish, without diet.

   Note: A bioassay requires 20 larvae per group and two groups per dose level. Two replicates at five dose levels, with two control groups, require a total of 240 second-instar larvae.

III. PREPARATION OF THE STOCK VIRUS SUSPENSION

A. Materials and equipment

1. Virus sample (dry powder)
2. Sterile buffered saline, prepared as follows:
   4.266 g BES (N, N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid);
   9.000 g NaCl;
   0.0075 g DOSS (dioctyl sulfosuccinate, sodium) *or* 100 µl of a 7.5% DOSS solution, such as Laboratory Aerosol® OT, Fisher Scientific Corporation;
   distilled water to 1,000 ml;
   adjust the pH to 7.13, at 20°C, with normal NaOH;
   autoclave (the pH will be 7.15 after autoclaving), *or* sterilize by filtration.
3. Sterile measuring cylinder (50-ml)
4. Sterile 125-ml Erlenmeyer flask, with aluminum foil closure
5. Sterile microspatula
6. Semi-micro Monel® blender vessel
7. Blender base with two-speed motor
8. Analytical balance (readability: at least 0.1 mg)

B. Procedure

1. Weigh 50 mg of virus preparation (dry powder) directly in a sterile 125-ml Erlenmeyer flask.
2. Add 50 ml of sterile buffered saline, cover with sterile aluminum foil, and mix well.
3. When the material is completely in suspension, usually within 5 minutes, pour the suspension into a sterile semi-micro Monel blender container.
4. Homogenize at low speed for 1 minute.
5. Pour the suspension in the original Erlenmeyer flask and cover. Refrigerate immediately.
6. Shake repeatedly before each use.

   Note: This suspension is designated as "stock suspension." *It contains 1 mg of preparation per milliliter of suspension.*

IV. PREPARATION OF SERIAL DILUTIONS OF THE STOCK SUSPENSION

A. Materials
1. Stock virus suspension
2. Sterile buffered saline (see III, A, 2)
3. Disposable sterile pipets (1-ml, 5-ml, and 10-ml)
4. Adhesive labels
5. Sterile 20-ml screw-cap vials

B_1. Procedure

This procedure should be used if an approximate estimate of the infectivity titer of the virus preparation is *not* available. This is a preliminary (or range-finding) assay.

1. Prepare five serial dilutions of the stock suspension in steps of tenths, in buffered saline:

Dilution	pg per µl	ng per cup (25 µl per cup)
1:25	40,000	1,000
1:250	4,000	100
1:2,500	400	10
1:25,000	40	1
1:250,000	4	0.1

2. Proceed to V and VI, then return to IV, B_2 when the results of the preliminary assay are available.

Note: Only 20 larvae per dose level will be needed for a preliminary assay (no replicates).

B_2. Procedure

This procedure should be used if an approximate estimate of the infectivity titer of the virus preparation is available, or if a preliminary LC_{50} has been determined by means of a range finding assay (B_1, above).

1. Prepare five serial dilutions of the stock suspension in steps of one-half, in buffered saline. These dilutions must be computed so that two of the dose levels will be above the preliminary or estimated LC_{50}, one at about the estimated LC_{50} level, and two below the LC_{50} level.

Example:
From the data gathered in an exploratory assay, we estimate that the LC_{50} is approximately 5 nanograms of preparation per cup. The following dilution steps will then be considered appropriate for a bioassay:

Dose level	ng per cup (25 µl per cup)	pg per µl	Dilution
1	20	800	1:1,250
2	10	400	1:2,500
3	5	200	1:5,000
4	2.5	100	1:10,000
5	1.25	50	1:20,000

V. INOCULATION OF THE TEST CUPS

A. Materials and equipment
1. Analyzer cups with diet (see I)
2. Racks for analyzer cups
3. Perforated caps for analyzer cups. Caps (Scientific Products diSPo beaker cap, polyethylene, B2716-1) are perforated with a hot needle, washed in distilled water, dried, placed in plastic petri dishes (20 per dish), and exposed in a UV sterilizer for 30 min.
4. Second-instar larvae (see II)
5. Dilute virus suspensions (see IV)
6. Sterile toothpicks
7. Infectivity test record charts (figs. 1 and 2)
8. 25-µl pipet with sterile tips
9. Sterile hood
10. Ultraviolet sterilizer
11. 30°C incubator

FSL Corvallis (Martignoni)

EVALUATE	COMPLETED	SUMMARY	COMPUTER	532

Purpose _____

Species _____ Stage, instar, mg _____

Source of insects _____ Gener. _____ Temp. _____ Diet _____

Inoculum _____ Volume _____ Route _____

Other data Container

No.	Date treat.	Inoculum	Date dead	S	P	Diagnosis	ST	L
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								

Dates checked and initials S= sacrificed ST= survival time (days) L= live at completion

					532

Figure 1.--Record chart for infectivity tests.

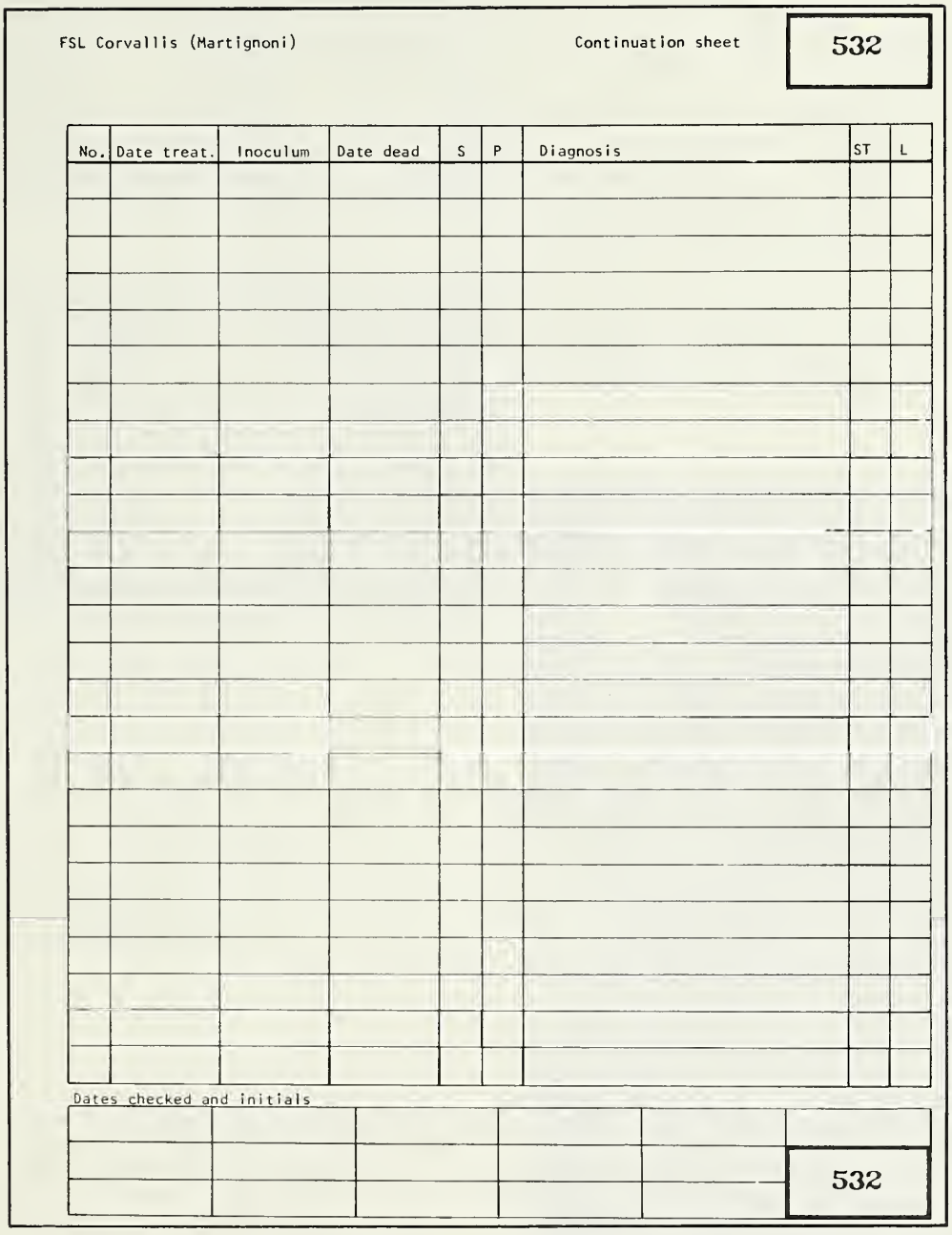

Figure 2.--Record chart for infectivity tests (continuation sheet).

B. Procedure

1. Remove the analyzer cups from the refrigerator at least two hours before the surface of the diet is to be inoculated.
2. Place 20 analyzer cups in each rack.
3. Number the cups (test number and cup number).
4. Place the racks with the cups under a sterile hood, for about 2 hours, to eliminate condensation droplets.
5. Shake vigorously the most dilute of the five suspensions and dispense 25 µl in each of five cups (fig. 3).

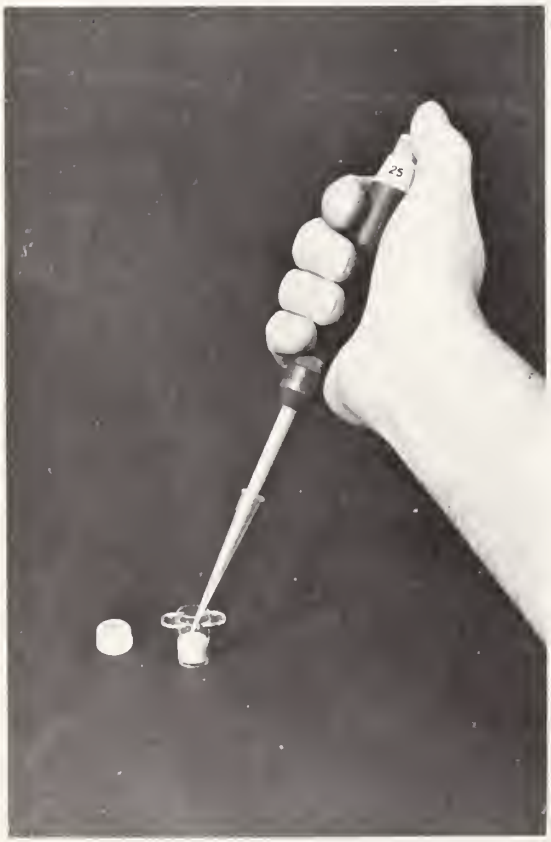

Figure 3.--Pipetting 25 µl of a dilute virus suspension on the diet surface in an analyzer cup.

6. Swirl the inoculum in the analyzer cups, making certain that the entire diet surface is wet.
7. Shake the suspension and proceed to dispense 25 µl to each of five additional cups. Swirl the inoculum.
8. Continue until 40 cups have been inoculated. Place the cups under a sterile hood (no light), at room temperature, for a minimum of 1 hour or until the diet surface is dry.
9. Repeat the above steps (5 to 8) with each of the other virus suspensions, in order of increasing concentration. For five serial dilutions, 200 cups will be needed. (Forty additional cups will serve as uninoculated controls.)
10. With a toothpick, transfer one second-instar larva to each cup and cap the cups with a perforated lid (figs. 4 and 5).
11. Place the cup racks on trays so that the cups are in horizontal position. Place the trays in a 30°C incubator, for 12 days, in darkness (fig. 6).
12. Complete the infectivity test record charts.

Note: Only 20 larvae per dose level will be needed for a preliminary assay (no replicates).

Figure 4.--Analyzer cup with 1 ml of diet 65-W-PEN and perforated cap.

Figure 5.--Capped analyzer cup with 1 ml of diet 65-W-PEN and 1 second-instar test larva.

Figure 6.--Trays, with cup racks and inoculated test cups (with larvae), in a 25°C incubator.

VI. EVALUATION

1. Daily, record the deaths on the infectivity test charts, during a 12-day observation period.
2. Usually the diagnosis (nucleopolyhedrosis) is readily apparent by macroscopic examination of the dead larva. In a few instances, however, a microscopic examination of larval tissues (to include epidermis, fat body, and tracheae) may be necessary. The cause of death is recorded on the infectivity test charts.
3. At the end of the 12-day observation period, tabulate the results as follows:

Dose ng per cup	Group	Number of test larvae	Number of dead larvae (nucleopolyhedrosis)	Mortality rate %
1.25	A	20	2	10
	B	20	1	5
2.50	A	20	4	20
	B	20	5	25
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.

4_a. If the data are from a preliminary (or range-finding) assay (IV, B_1) and the mortality rate for two of the five dose levels falls between 10 and 90 percent, draw a line through the two points, on Berkson's logarithmic logistic ruling graph paper (Codex No. 32-450 or 32-454). Read the LC_{50} (ng/cup) from the plotted line. If only one point falls between 10 and 90 percent, draw a line through this point, with a slope of 3. Read the LC_{50} from the plotted line. Resume IV, B_2.

4_b. If the data are from a conclusive assay (IV, B_2), compute the LC_{50} on Berkson's graph paper (see above) or by minimum logit chi-square analysis (Berkson 1953; LOCSAN program by R. L. Giese, Purdue University). The program's printout lists the LC_{50} (ng/cup), the 95-percent fiducial limits, the slope of the regression line, and tests for linearity and homogeneity of response among the test insects.

VII. CHECKLIST OF ITEMS NEEDED FOR ONE BIOASSAY

Aluminum foil
Aspirator, rubber, for pipets
Balance, analytical
Blender, semi-micro
Blender base, two-speed motor
Caps, perforated, for analyzer cups (240)
Coverslips
Cups, analyzer, 2-ml (240)
Cylinder, measuring, 50-ml, sterile
Diet, insect, 65-W-PEN (400 ml)
Dishes, petri, 100- x 15-mm, disposable (24)
Flask, Erlenmeyer, 125-ml, sterile
Graph paper, logarithmic logistic ruling
Hot plate
Incubator, 30°C
Instruments, dissecting (set)
Hood, sterile
Labels, adhesive (24)
Larvae, second-instar (240)
Microscope
Paper tissues, sterile
Pipet, 25-microliter
Pipets, 1-ml, sterile, disposable (3)
Pipets, 5-ml, sterile, disposable (6)
Pipet, 10-ml, sterile, disposable
Pipet, 10-ml, wide-tip, sterile, disposable
Racks, for analyzer cups (12)
Record charts, infectivity test (one initial sheet, nine continuation sheets)
Refrigerator
Saline, buffered, sterile (100-ml)
Slides, microscope (box)
Spatula, micro, sterile
Sterilizer, UV
Thermometer, 60°C
Tips, for microliter pipet, sterile (12)
Toothpicks, sterile (box)
Trays, for analyzer cup racks (1 ten-rack, 1 two-rack)
Vials, screw-cap, 20-ml, sterile (7)
Virus preparation (50 mg)
Water bath

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KEYWORDS: Bioassay, nucleopolyhedrosis, virus (insect), virus (-insecta, virus (-forest pest control, insect diseases, diseases (insect), insect damage control (forest), biological control (-forest pests, Douglas-fir tussock moth, *Orgyia pseudotsugata*.

The mission of the PACIFIC NORTHWEST FOREST AND RANGE EXPERIMENT STATION is to provide the knowledge, technology, and alternatives for present and future protection, management, and use of forest, range, and related environments.

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